Endoplasmic reticulum-associated degradation of the NR1 but not the NR2 subunits of the N-methyl-D-aspartate receptor induced by inhibition of the N-glycosylation in cortical neurons

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Running title: NR1 is an ERAD substrate

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Abstract

The N-Methyl-D-Aspartate receptor (NMDAR) is fundamental to normal and pathological functioning of neurons. The receptor subunits are N-glycosylated proteins synthesized in the endoplasmic reticulum (ER) which fold, mature and oligomerize as they transit through the secretory pathway. Although the early processes of biogenesis are fundamental to NMDAR expression and function, our knowledge of them is nevertheless limited. Additionally, the investigation of NMDAR synthesis is highly relevant because ER dysfunction, frequently associated to acute and degenerative brain diseases, might alter this process.

We characterize here the effect of ER stress produced by inhibition of N-glycosylation on NMDAR synthesis and function. We use first heterologous systems of NMDAR expression where NR1 and NR2A subunits are synthesized in non-neuronal cells. The function of these NMDARs as Ca^{2+} channels is repressed by tunicamycin, due to the inhibition of NR1, but no NR2A, synthesis. The regulation of NR1 is relevant to central nervous system since a dramatic decrease in synthesis of this subunit and assembly of NMDARs is observed in cortical neurons treated with tunicamycin. The inhibition of NR1 synthesis is not due to changes in levels of mRNA but associated to the earliest stages in NMDAR biogenesis. The inhibition of N-glycosylation activates ER specific stress responses in neurons, which include the ER-associated degradation (ERAD), mechanism responsible for differential and extremely efficient degradation of nonglycosylated NR1 by the proteasome after ubiquitination. Since this is an obligatory NMDAR component, the significant sensitivity of NR1 to ER stress will have important consequences on receptor function.

Keywords: NMDA receptors; ER stress; ERAD; N-glycosylation; quality control; tunicamycin

Introduction

The N-Methyl-D-Aspartate type of glutamate receptor (NMDAR) plays crucial roles in neuronal plasticity, learning and memory, fundamentally due to its high Ca^{2+} permeability (Mori and Mishina 1995). Physiological levels of synaptic NMDAR activity are also extremely important for neuronal survival (Hardingham et al. 2002; Papadia et al. 2005), while agonist overstimulation can induce excitotoxic cell death and contribute to neuronal degeneration in different disorders (Choi 1988). Thus, the regulation of NMDAR expression and function is fundamental to maintain or adjust neuronal excitability to changing demands, avoiding neuronal dysfunction and death.

The NMDARs are hetero-oligomeric proteins formed by obligatory NR1 subunits interacting with NR2A-D, conferring functional variability (Cull-Candy and Leszkiewicz 2004). The subunits are N-glycosylated proteins synthesized in the endoplasmic reticulum (ER). The earliest events in NMDAR biogenesis, including N-glycosylation, folding, carbohydrate maturation, oligomerization and transport to the plasma membrane take place along the secretory pathway. Although critical to NMDAR expression and function, our knowledge of them is nonetheless limited. A well-established feature is the different trafficking of NR1 and NR2 subunits. Approximately 50-60% of NR1 does not reach the cell surface (Hall and Soderling 1997) and constitutes an intracellular pool of immature protein of very short half-life compared to NR2A-assembled NR1 reaching the cell surface (Chazot and Stephenson 1997; Garcia-Gallo et al. 1999; Huh and Wenthold 1999).

Interestingly, ER dysfunction is observed in cerebral ischemia and other acute and neurodegenerative diseases (Paschen and Mengesdorf 2005). In addition, congenital disorders of glycosylation (CDGs), a group of inherited disorders caused by defects in synthesis and processing of N-glycans, are associated to impaired central nervous function in nearly all cases (Jaeken 2003; Freeze 2006). A possibility is that the earliest events in the biogenesis of key proteins in the central nervous system, such as the NMDAR, might be modified by ER malfunction and contribute to neuronal damage.

Given its central role in protein synthesis and calcium homeostasis, different signaling pathways have evolved to preserve ER function in a stress situation, for example due to accumulation of misfolded proteins. These pathways are collectively known as the "unfolded protein response" (UPR) (Kaufman 1999) or ER stress response. They consist in the inhibition of protein synthesis (Harding et al. 2000), reducing the work-load imposed on the folding machinery, and transcriptional induction of genes encoding ER chaperones and proteins involved in different steps in the secretory pathway (Casagrande et al. 2000; Gething and Sambrook 1992; Kozutsumi et al. 1988; Travers et al. 2000). The ER also prevents misfolded or misassembled proteins from moving along the secretory pathway by another response known as the quality control system. Defective proteins are retained in the ER and, eventually, degraded by a mechanism known as ER-associated degradation (ERAD) (Bonifacino and Weissman 1998) that requires retrotranslocation to the cytosol and proteasomal degradation following ubiquitination (Brodsky and McCracken 1999). Finally, in the case of persistent or intense stress, the ER can also trigger apoptosis (Boyce and Yuan 2006).

In this investigation we characterize the susceptibility of the NMDAR to ER stress induced by inhibition of N-glycosylation. To this aim, we analyze the effect of the suppression of N-glycosylation by tunicamycin on recombinant NMDARs or those produced by cortical neurons. We show that treatment with this antibiotic inhibits NMDAR function. In the context of the ER stress response induced in neurons by tunicamycin, we identify NR1 as a NMDAR subunit differentially sensitive to ER stress, and show it to be a substrate of the ERAD mechanism. Since NR1 is an essential receptor component, degradation of newly synthesized protein will have a great impact on NMDAR expression and function.

Materials and Methods

Chemicals - NMDA receptor antagonist 2-amino-phosphono-pentanoic acid (DL-AP5) and MK-801 were from Tocris-Cookson (Bristol, UK). Tunicamycin, N-acetyl-leucyl-leucyl-norleucinal (calpain inhibitor I, herein ALLN), N-acetyl-leucyl-leucyl-methional (calpain inhibitor II, herein ALLM), brefeldin A, cytosine β-D-arabinofuranoside (AraC), poly-L-lysine, and L-laminin were all from Sigma Co (St. Louis,

MO). N-glycosydase F was from Roche Molecular Biochemical (Mannheim, Germany). MG132 and clasto-lactacystin-ß-lactone were purchased from Calbiochem Novabiochem. $^{45}CaCl_2$ (36 mCi/mg) was obtained from DuPont NEN. All other products were of the highest quality available.

Cell culture – Human embryonary kidney cells HEK293 (ATCC CRL 1573) were grown in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal bovine serum, 40 µg/ml gentamicin and 2 mM glutamine. African green monkey kidney cells (BSC-40) were grown as before but using newborn calf serum. Cells were grown at 37°C in 5% CO_2. Vaccinia virus was manipulated in the P2 facility of the Instituto de Investigaciones Biomédicas. For immunofluorescence experiments, HEK293 were grown on coverslips previously treated with 0.2% gelatin for 2 h at 37°C, and then washed and dried under sterile conditions. Primary culture of embryonic rat neurons was performed basically as described (Rose et al. 1993) with some modifications. Plates were treated with poly-L-lysine (100 µg/ml) and laminin (4 µg/ml) overnight at 37°C before seeding. Cerebral cortices from 18-days old rat embryos (Wistar) were dissected and mechanically dissociated in culture medium (Eagle minimum medium supplemented with 28.5 mM $NaHCO_3$, 22.2 mM glucose, 0.1 mM glutamine, 5% FBS and 5% donor horse serum). Cells were seeded at a density of $0.3x10^6$ cells/cm^2 in the same media. To inhibit growth of glial cells, AraC (10 µM) was added to the culture at day 12 and kept for the next 48 h.

Plasmids and viruses - Plasmids coding for NR1-1a splice variant (Moriyoshi et al. 1991) and NR2A (Ishii et al. 1993) subunits of the rat NMDAR were kindly supplied by Dr. S. Nakanishi. The plasmid containing a dog calnexin cDNA was provided by Dr. D. Williams (University of Toronto, Ontario, Canada). All these cDNAs were cloned in pBlueScript (Stratagene, La Jolla, CA). Dr. A. S. Lee (University of Southern California, Los Angeles, USA) gave us plasmids pTZSSB-BiP and pTZ-CHOP containing hamster cDNAs, respectively coding for chaperone grp78/BiP and transcriptional factor CHOP/GADD153. Plasmid YFP-NR1, generously provided by Dr. J. Luo (Zhejiang University School of Medicine, China), encodes the NR1-1a subunit containing the yellow florescence protein (YFP) inserted between the third and fourth codons after the predicted sequence for the signal peptide (Qiu et al. 2005). Plasmid pME18S-HA-

NR2A was a generous gifts of Dr. T. Yamamoto (University of Tokyo, Japan) and contain the rat NR2A cDNA with a hemagglutinin epitope in the N-terminus between amino acid residues 51 and 52 (Hironaka et al. 2000). The recombinant VV vTF7-3 expressing the T7 RNA polymerase gene under the control of the virus early/late promoter p7.5 (Fuerst et al. 1987) was grown and titrated in BSC-40 cells according to standard procedures.

Heterologous expression of recombinant NMDAR - HEK293 cells (3 x 10^6, in 6-well plates) were infected with vTF7-3 virus at a multiplicity of 4 plaque forming units (pfu) per cell for 1 hr at 37°C. After adsorption, virus inoculum was removed, cells were washed once with DMEM with no serum and transfected using Transfectace liposomes as described (Garcia-Gallo et al. 1999). Transfection of NR1 and NR2A cDNAs was performed with 4 μg each plasmid. Control transfections were done replacing these plasmids by pBlueScript SK (-) as required. Lipids and DNA were incubated for 15 min in DMEM without serum and then added to the infected cells for 5-6 h. Then, cells were fed with DMEM containing 10% serum, 2 mM DL-AP5, a competitive antagonist used to prevent NMDAR activation by glutamate present in the culture media, and 2 μg/ml tunicamycin, as indicated. Incubation proceeded up to 24 h after infection before further treatment. Alternatively, HEK293 cells (2 x 10^5, in 24-well plates) grown on coverslips were transfected with 0.5 μg of plasmids YFP-NR1 and pME18S-HA-NR2A using standard calcium phosphate methods. After 5 h of transfection, cells were washed and fed with DMEM plus 10% fetal bovine serum, 2 mM DL-AP5 and 2 μg/ml tunicamycin, as indicated. Incubation proceeded for another 24 h before fixation.

Calcium influx through the NMDA receptor - Cells ($0.8x10^6$) were infected/transfected as before with or without tunicamycin. Twenty-four hours after infection, they were washed three times with 120mM NaCl, 5.4mM KCl, 0.8 mM $MgCl_2$, 15mM glucose, 20mM Hepes (pH 7.4), 10mM NaOH, 0.002% Phenol Red (HCSS medium) (Rose et al. 1993), containing 1.8 mM $CaCl_2$ and 10 μM glycine. Then, cells were incubated for 5 min at room temperature in 250 μl of the same medium with the addition of 1mM NMDA and 1.25 μM $^{45}CaCl_2$ (the final concentration in the assay was 5 μCi/ml), with or without 10 μM MK-801 as indicated (Lu et al. 1996). Cells were then washed four times in HCSS/calcium/glycine medium as before,

and lysed with 0.4 ml of 2% SDS with occasional swirling for 20 min at room temperature before counting radioactivity.

Metabolic labeling - Cells were starved in DMEM medium without methionine nor cysteine (Biowhittaker) for 3 h in the presence of 2 mM (HEK293 cells) or 200 μM DL-AP5 (neurons). Then, they were pulsed for 30 min (HEK293 cells) or labeled for 4 h (neurons) using 150 μCi/ml of ^{35}S-Methionine. The time of labeling for neurons was established as the minimum required for an efficient detection of the subunits. After that, cells were washed in cold PBS before lysis in 1% NP-40, 20 mM Tris-Cl (pH 8), 80 mM NaCl, and 20 mM EDTA (HEK293 cells) or RIPA buffer (10 mM Na_2HPO_4, pH 7.2, 150 mM NaCl, 1% sodium deoxicolate, 1% NP-40, 0.1% SDS) (neurons) for 30 min at 4°C. Lysis buffers contained protease inhibitors at the following concentrations: 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 mM benzamidine. When indicated tunicamycin (2 μg/ml), brefeldin A (5 μg/ml), MG132 (20 μM), ALLN (50 μM), ALLM (50 μM) and lactacystin (50 μM) were added during starvation and maintained during the time of labeling.

Immunoprecipitation - HEK293 and neuron protein extracts were diluted respectively with one or two volumes of their corresponding lysis buffers but lacking detergents. Approximately 200 μg of protein were incubated with 2.5 μg of a mouse monoclonal antibody specific for the NR1 subunit (aminoacids 660 to 811) (Pharmingen, San Diego, CA), or 0.5 μg of rabbit polyclonal antibodies specific for the C-terminus of NR2A and 2B subunits or calnexin (Chemicon, Temacula, CA). After 1h at 4°C, immunocomplexes were precipitated with 100 μl of 10% Protein A Sepharose also for 1 h at the same temperature. Beads were washed four times with the buffer used for immunoprecipitation before solubilization in sample buffer and gel fractionation. Detection of the immunoprecipitated proteins was performed by autoradiography of dried gels or immunoblotting, with the same antibody used for immunoprecipitation or a different one.

Immunoblot analysis – Cortical cultures were lysed in RIPA buffer containing protease inhibitors as before. Protein determination was performed using the BCA reagent from Pierce (Rockford, IL). Equal

amounts of total extracts (25-50 μg) or proteins immunoprecipitated as described were fractioned by polyacrylamide gel electrophoresis and transferred to PVDF membranes (Pall, Life Sciences). Immunodetection of proteins was performed by standard procedures using the antibodies mentioned above as well as mouse monoclonal antibodies for the pro-apoptotic transcriptional factor CHOP/GADD153 (Santa Cruz Biotechnology, Santa Cruz, CA), a rat monoclonal antibody specific for caspase-12 generously provided by Dr. J. Yuan (Harvard Medical School, USA) (Nakagawa et al. 2000) or rabbit polyclonal antibodies that recognize calnexin (Chemicon, Temacula, CA) or ubiquitin (DakoCytomation, Denmark). Goat secondary antibodies coupled to horseradish peroxidase were from Santa Cruz Biotechnology (Santa Cruz, CA). Immunocomplexes were detected with the Bioluminescence kit from Perkin-Elmer Life Sciences (Boston, MA).

Immunofluorescence – Transfected HEK293 were fixed in 4% (w/v) paraformaldehyde in PBS for 2 min at 4^0C and washed with PBS. Then, cells were permeabilized and blocked for 30 min at room temperature in 10% (v/v) horse serum, 0.1% (v/v) Triton X-100 in PBS. Dilution of the antibodies was performed in 10% (v/v) horse serum and cells were incubated for 1 h at room temperature as indicated. We used a rabit antibody for GFP (Invitrogen, Carlsbad, CA) and a goat polyclonal sera for NR2A (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactivity was detected with secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, Eugene, OR) as indicated, before mounting with Fluoromount-G (SouthernBiotech). Confocal images were acquired using a Radiance 2000 confocal (BioRad) coupled to an inverted Axiovert S100 TV microcope (Zeiss) with a 63x Plan-Apochromat oil immersion objective. Overlaying images produced the two-color merged image.

Northern blot analysis - Total RNA was prepared using "TriReagent" (Sigma Co., St. Louis, MO) according to the manufacturer's instructions. Approximately 10 μg of total RNA were fractionated on 1% agarose gels prepared in 20 mM MOPS buffer, pH 7.2, 0.6% formaldehyde. After transfer to Nylon membranes, the RNA was hybridized to DNA probes corresponding to nucleotides 344 to 1280 of the NR1-

1a splice variant, or cDNAs encoding calnexin, BiP/grp78 and CHOP/GADD153 as described, labeled by standard procedures.

Results

Calcium influx mediated by recombinant NMDARs is suppressed by inhibition of N-glycosylation - We expressed recombinant NMDARs very efficiently in non-neuronal cells using a vaccinia virus-regulated system and transfection of the cDNAs encoding subunits NR1 and NR2A (Garcia-Gallo et al. 1999). The NMDARs induced a significant net accumulation of $^{45}Ca^{2+}$ in HEK293 cells treated with the receptor co-agonists NMDA and glycine (0.83 nmoles/5 min), which was significantly reduced in the presence of the NMDAR antagonist MK-801 (0.12 nmoles/5 min; $p<0.01$) (Fig. 1). Only receptors formed by NR1 and NR2A subunits were capable to induce $^{45}Ca^{2+}$ accumulation upon activation, while homomeric NR1 receptors could not. Once demonstrated that heteromeric NMDARs expressed in this way are functional and respond to specific receptor agonists and antagonists, we analyzed the effect of the inhibition of N-glycosylation on NMDAR function. The net accumulation of $^{45}Ca^{2+}$ was significantly reduced by 2 μg/ml of tunicamycin (0.21 nmoles/5 min) compared to untreated cells ($p<0.01$). These low levels of $^{45}Ca^{2+}$ influx were close to those obtained in the presence of MK-801, or in cells expressing the NR1 subunit alone. In conclusion, the results presented in this experiment demonstrate that N-glycosylation of the receptor subunits is required for NMDAR function.

Synthesis of recombinant NR1 subunits is differentially blocked by inhibition of N-glycosylation – The inhibition of N-glycosylation frequently affects stability and trafficking of proteins in the secretory pathway, although this is not a universal fact. To characterize the mechanism responsible for the lack of NMDAR function in the presence of tunicamycin, we first analyzed *de novo* synthesis of the receptor subunits by a 30 min pulse with ^{35}S-methionine (Fig. 2A). Before pulse, cells were pretreated for 3 h with tunicamycin, a time established as the minimum required for complete blockage of N-glycosylation without general inhibition of protein synthesis as part of the UPR (Harding et al. 2000). Synthesis of the subunits was then analyzed by immunoprecipitation with specific antibodies (herein IPαNR1 and IPαNR2) followed by

autoradiography of the ^{35}S-labeled proteins. In tunicamycin-treated cells, nonglycosylated (ng) NR2A (M_r 165-kDa) was the sole *de novo* synthesized NR2A while the untreated cells produced similar levels of the glycosylated form of this subunit (M_r 180-kDa) (Fig. 2A, lower panel). Shorter times of tunicamycin pretreatment resulted in mixed populations of NR2A and ngNR2A subunits (data not shown). Contrary to that, we detected the glycosylated form of NR1 (M_r 116-kDa) in the untreated cells (Fig. 2A, upper panel) but no protein corresponding to ngNR1 (M_r 97-kDa) after tumicamycin treatment. Immunoblot analysis (IB) of the immunoprecipitated proteins (Fig. 2B) showed that this short treatment with tunicamycin however had no effect on the steady-state levels of the subunits, and that the total amount of ngNR2A was still too low to be detected in this way. The monoclonal NR1 antibody recognizes a sequence present in the different NR1 isoforms and, then, they are all regulated by tunicamycin. Since that sequence includes two of the NR1 consensus N-glycosylation sites, there was a possibility that the antibody could not immunoprecipitate ngNR1 subunits. Therefore, total extracts were deglycosylated *in vitro* with N-glycosydase F before immunoprecipitation and immunoblot analysis with the NR1 antibody (Fig. 2C), finding that deglycosylated and glycosylated NR1 were similarly recognized. All together, these results strongly suggest that the selective blockage in the synthesis of the NR1 isoforms, obligatory subunits of the NMDAR, causes the loss of the receptor function.

Interestingly, *de novo* synthesis of NR2A, also an N-glycosylated protein, is not affected by the inhibition of N-glycosylation. However, transport of newly synthesized NR2 subunits to the cell membrane has been shown to require synthesis of NR1 (Fukaya et al. 2003). Therefore, we further investigated the destiny of ngNR2A by immunofluorescence of permeabilized HEK293 cells after standard transfection of cDNAs encoding tagged-subunits (Fig. 2D). In the untreated cells, we observed a very good co-localization of the NR1 and NR2A proteins in the plasmatic membrane while ngNR1 became hardly detectable in tunicamycin-treated cultures. On the contrary, we detected high levels of ngNR2A but this protein was not transported to the membrane and formed intracellular aggregates. These results, obtained using an alternative heterologous system, not only allowed confirmation of previous observations but also discarded

the possibility that NR1 regulation induced by inhibition of N-glycosylation was helped by subunit over-expression in the virus-regulated system.

Blockade on NR1 but no NR2 synthesis is induced in cortical neurons by inhibition of N-glycosylation – The results obtained in the heterologous systems suggested that the earliest events in the synthesis of the NMDAR might be affected by inhibition of N-glycosylation in the central nervous system. To investigate this possibility, we analyzed primary cultures of rat embryonic cortical neurons of 14 days *in vitro* (DIVs 14), when *de novo* synthesis of NMDAR subunits is very active (data not shown). The main NR2 subunits expressed in cortical neurons at this time in culture are NR2A and NR2B (herein NR2), which have similar molecular weight and cannot be distinguished by antibodies directed to their conserved regions. We first performed metabolic-labeling with ^{35}S-methionine in the presence or absence of tunicamycin (4 h), and analyzed synthesis of the NMDAR subunits by immunoprecipitation with specific antibodies and autoradiography (Fig. 3A). Quantitation of the results obtained in three independent experiments (Fig. 3B) demonstrated that levels of ^{35}S-labeled ngNR1 were reduced by 77% in the treated neurons compared to N-glycosylated subunits expressed in the untreated cultures ($p<0.001$). Contrary to that, levels of ngNR2A and ngNR2B (ngNR2) in cells treated with tunicamycin were only modestly reduced (28%) compared to those of the untreated cells, probably due to general inhibition of protein synthesis as will be shown below. However, the difference in the relative levels of ^{35}S-labeled ngNR1 and ngNR2 was significant ($p<0.001$). Immunoblot analysis of the immunoprecipitated proteins showed that this short treatment with tunicamycin (7 h) had no effect on the steady-state levels of NR2 (Fig. 3C) or NR1 (Fig. 3D), and only a small amount of ngNR2 could be detected in the treated neurons although the glycosylated form was still the most abundant (Fig. 3C). Longer treatments with tunicamycin ($\geq$ 24 h) were needed to observe modest decreases in the steady-state levels of NR1 subunit (data not shown). From these experiments, we conclude that there is a specific blockage of NR1 synthesis induced by inhibition of N-glycosylation, which does not occur for the other subunits forming the NMDAR in cortical neurons.

Disruption of NMDAR assembly in cortical neurons induced by inhibition of N-glycosylation – The interference with N-linked glycosylation may disrupt other processes occurring in the secretory pathway and, therefore, we next analyzed if that is true for the NMDAR. Cortical cultures were labeled as before in the presence or absence of tunicamycin and/or brefeldin A, a drug causing the collapse of Golgi stalks on the ER, thus inhibiting processes in the secretory pathway taking place beyond the ER (Klausner et al. 1992). We performed immunoprecipitations in conditions compatible with co-immunoprecipitation of interacting proteins (Fig. 4). The immunoprecipitation of NR1 not only showed the expected decrease in levels of newly synthesized ngNR1 in tunicamycin-treated cells, but also in neurons treated with brefeldin A together with tumicamycin (Fig. 4A). Therefore, the inhibition of NR1 synthesis is a process previous to export from the ER to the Golgi compartment. The pattern of NR1 observed in the control cells, characteristic of N-glycosylated proteins, was simplified in neurons treated with brefeldin A (Fig. 4A) suggesting that the lower-mobility protein probably corresponds to a form of NR1 that needs exit of the ER for carbohydrate maturation. Anyway, NR2 was co-immunoprecipitated with both maturation forms of NR1 demonstrating that interaction between the NMDAR subunits is already established in the ER. However, assembly of the NMDAR required N-glycosylation, as shown by the absence of interaction between ngNR2 and ngNR1 (Figs. 4A and 4B). As before, synthesis of NR2 was not modified by tunicamycin (Fig. 4B), and only a small fraction of newly synthesized NR1 interacts with NR2 in the untreated cells (compare Figs. 4A and 4B), as previously described (Huh and Wenthold 1999). In conclusion, these results demonstrate the existence of a mechanism induced by the inhibition of N-glycosylation causing a significant decrease in the synthesis of NR1 subunits and also in the assembly of heteromeric NMDARs in the ER compartment.

Inhibition of NR1 synthesis induced by tunicamycin is not due to changes in mRNA levels - To further characterize the mechanism responsible for the inhibition of NR1 synthesis, we next compared by Northern blot analysis the levels of NR1 mRNA in neurons treated or not with tunicamycin for a time known to block *de novo* synthesis of this protein (7 h) (Fig. 5A). We found no significant variation in the NR1 mRNA levels normalized to rRNA, used as a control. Quantitation of three independent experiments showed a 97% +/- 8.3

of the NR1 mRNA levels remaining after 7 h of treatment, compared to the untreated neurons. Longer treatments with tunicamycin (24 h) were required to observe reductions in the amounts of NR1 mRNA (Fig. 5A), although they were no specific. Similar decreases were also produced for calnexin mRNA or 28S and 18S rRNAs, and they are probably due to apoptosis associated to prolonged ER dysfunction. In conclusion we show that, before a general degradation of RNA takes place, there is a specific mechanism on NR1 synthesis induced by inhibition of N-glycosylation, which cannot be explained by a decrease in the levels of its encoding mRNA.

Induction of an ER stress response in cortical neurons by inhibition of N-glycosylation – The results presented so far suggested that inhibition of NR1 synthesis might be associated to ER stress responses induced by tunicamycin in cortical neurons. To investigate the activation of these signaling cascades, we first analyzed the transcriptional induction of genes important for the UPR (Fig. 5A). In neurons treated for 7 h with tunicamycin, we demonstrated the induction of the mRNA for the ER chaperone grp78/BiP which resulted in a corresponding increase in protein levels (data not shown). Similarly, the mRNA encoding for the pro-apoptotic transcriptional factor CHOP/GADD153 was induced (Fig. 5A), contributing to a significant increase in levels of this protein in neurons treated with tunicamycin (Fig. 5B, upper panel). These inductions were specific for stress-related genes and not observed for other mRNAs or proteins such as calnexin (respectively, Fig. 5A and Fig. 5B, lower panel). The UPR also results in a general inhibition of protein translation and, therefore, we measured the ^{35}S-methionine incorporation as trichloro-acetic acid precipitable protein. We found a 25.3% inhibition of *de novo* synthesis in neurons treated with tunicamycin for 7 h compared to the untreated cells.

Finally, persistent or intense ER stress can also trigger apoptosis, a process where activation of pro-caspase-12 has a leading role (Nakagawa et al. 2000). Our results showing rRNA degradation (Fig. 5A) or induction of CHOP/GADD153 (Fig. 5B, upper panel) strongly suggested apoptosis induction in cortical neurons after prolonged inhibition of the N-glycosylation. We confirmed this point by characterizing the activation of pro-caspase-12 (Fig. 5B, middle panel), observing a processing indicative of enzyme activation

after 7 h of tunicamycin treatment, although activation dramatically increased afterwards. In conclusion, all together these results demonstrate that blockage of N-glycosylation in primary cultures of cortical neurons activates ER specific signaling pathways directed to promote cell survival or, after persistent inhibition, apoptosis.

Nonglycosylated NR1 subunits are differentially degraded by the proteasome in cortical neurons – Among the adaptative pathways constituting the ER stress response, the activation of proteasome-dependent ERAD is fundamental to remove misfolded or unassembled proteins from the ER, and alleviate the burden on this organelle. Thus, we investigated if ngNR1 might be a specific substrate of the ER quality control system in neurons treated with tunicamycin. In that case, ngNR1 would be retrotranslocated to the cytoplasm, ubiquitinated and degraded by the proteasome soon after synthesis. We analyzed as before *de novo* synthesis of NR1 in neurons treated with tunicamycin and different inhibitors of the proteasome (Fig. 6). We first used MG132, a reversible inhibitor of the proteasome, and observed that the levels of ngNR1 significantly increased in neurons treated with tunicamycin together with MG132 compared to cells only incubated with the antibiotic (Fig. 6A, upper panel). Quantitation of the results (Fig. 6B) showed that synthesis of NR1 was only reduced a 40% in those neurons, compared to a 77% inhibition found in cultures treated with tunicamycin alone, being these differences significant ($p<0.001$). As shown before for NR2, inhibition of N-glycosylation only had minor effects on the synthesis of calnexin, which were independent of the presence of MG132 (Fig. 6A, lower panel). Next, we established that the ngNR1 subunit immunoprecipitated in neurons treated with tunicamycin and MG132 was ubiquitinated, since an antibody specific for ubiquitine recognized it in immunoblot (Fig. 6C).

Given that MG132 has been reported to be also active on neutral cystein proteases such as calpain (Tawa et al. 1997), we decided to analyze other proteasome inhibitors. We used ALLN, a proteasomal inhibitor when employed at high concentrations (50 μM) or, as a negative control, ALLM, a related peptide of 50-100-fold lower potency (Fig. 6D). Combined treatment with tunicamycin and ALLN notably increased the amount of ^{35}S-labeled ngNR1 compared to cells treated with tunicamycin and ALLM, or with antibiotic

alone (Fig. 6D, upper panel). Finally, by using the specific proteasome irreversible inhibitor lactacystin (Fig. 6D), we also observed a significant increase in levels of ngNR1 in cells incubated with tunicamycin and lactacystin compared to cultures treated with the antibiotic alone. As seen before, levels of the NR2 subunits were nearly equivalent in the different conditions tested (Fig. 6D, lower panel). Therefore, these results demonstrate that the activity of the proteasome is required for the decrease in levels of ngNR1 induced by inhibition of N-glycosylation and that ER-associated NR1 degradation is the mechanism responsible for it.

Discussion

Considering the central role of the NMDAR in the physiopathology of the central nervous system, and given that the receptor subunits are synthesized, modified and assembled in the ER, it was essential to investigate the effect of altered ER function on NMDAR expression and functioning. In this investigation, we have analyzed the consequences of ER dysfunction induced by the inhibition of N-glycosylation on recombinant NMDARs produced in heterologous expression systems and in the receptors expressed in primary cultures of rat cortical neurons. The function of the NMDAR as Ca^{2+} channel is significantly inhibited by tunicamycin in a vaccinia virus-regulated heterologous system, explaining our previous observations showing a dramatic decrease in cell death due to NMDAR overactivation after treatment with this antibiotic (Garcia-Gallo et al. 1999). The suppression of N-glycosylation frequently affects the function of proteins by modifying their stability and trafficking in the secretory pathway. However, the role of this modification must be examined for each protein since is not universal and may be different even for related proteins (Diop and Hrycyna 2005). In the case of the NMDAR, the mechanism responsible for the inhibition of function is the blockade of NR1 synthesis induced by suppression of N-glycosylation. Contrary to that, synthesis of NR2A, likewise a N-glycosylated subunit, is not affected by the inhibition of N-glycosylation, although ngNR2A cannot be transported to the cell membrane and forms intracellular aggregates.

A similar response of NR1 subunits to ER dysfunction can be also observed in cortical neurons briefly treated with tunicamycin (7 h). Subunit NR1 has a differential sensitivity to inhibition of N-glycosylation

compared to NR2A and NR2B, and newly synthesized NR1 mostly disappears before exit of the ER. In addition to that, the limited ngNR1 protein expressed in neurons treated with tunicamycin is not able to establish interactions with ngNR2 subunits and, therefore, to form heteromeric NMDARs. These results, together with the observation that levels of NR1 mRNA are not affected by short tunicamycin treatment, strongly suggest that misfolding of proteins due to inhibition of N-glycosylation might induce specific ER stress responses and differential degradation of ngNR1 but not the ngNR2 proteins. In fact, activation of ER stress responses of the UPR type could be demonstrated in neurons treated with tunicamycin and, in such cellular context, degradation of ngNR1 takes place with the participation of the ubiquitin/proteasome system showing ngNR1 as a specific substrate for the ERAD response. However, given that proteasomal inhibition does not completely prevents ngNR1 degradation, we cannot exclude the participation of other mechanisms in NR1 regulation by ER stress, such as a specific blockage of mRNA translation or the disposal of defective subunits by proteolytic systems different to the proteasome (Frigerio and Lord 2000). It will be interesting to investigate if these alternative mechanisms are really involved in NR1 regulation.

The data presented here and our previous results (Garcia-Gallo et al. 2001) clearly establish NR1 as a protein highly dependent on N-glycosylation for stability and assembly. Since transport of newly synthesized NR2 subunits to the cell membrane requires synthesis of NR1 as demonstrated in a NR1 knockout mice (Fukaya et al. 2003), it is very probable that inhibition of NR1 synthesis by tunicamycin will cause the intracellular retention of newly synthesized NR2 subunits similarly to the heterologous system. Being NR1 an obligatory NMDAR component, the result will be a progressive decrease in the levels of synaptic and extra-synaptic NMDARs. Nonetheless, the molecular mechanisms responsible for the differential sensitivity of NR1 and NR2A/B subunits to ER stress are still unknown. The events leading to the sorting and targeting of specific substrates to the translocation pore for their degradation are not clear.

What is the relevance of our results to brain physiology? Several data in the literature point to the induction of ER stress in neurons in some physiological and pathological situations of the central nervous system (Paschen and Mengesdorf 2005). Additionally, congenital defects in the biosynthesis of N-linked

glycans cause severe multisystemic disorders with frequently impaired central nervous function (Jaeken 2003; Freeze 2006). In these situations, the earliest events in the biogenesis of critical molecules in functioning of the central nervous system, such as the NMDAR, might be inhibited. Since the synaptic NMDARs are fundamental for neuronal survival in the adult brain (Hardingham et al. 2002; Papadia et al. 2005), a decrease in receptor function due to NR1 degradation as a consequence of ER stress might be contributing to neuronal injury.

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Abbreviations: ALLM, N-acetyl-leucyl-leucyl-methional (calpain inhibitor II); ALLN, N-acetyl-leucyl-leucyl-norleucinal (calpain inhibitor I); AraC, cytosine β-D-arabinofuranoside; DL-AP5, 2-amino-phosphonopentanoic acid; ER, endoplasmic reticulum; ERAD, ER-associated degradation; NMDA, N-Methyl-D-Aspartate; UPR, unfolded protein response.

Figure legends

Figure 1. *Inhibition of calcium influx mediated by a recombinant NMDAR after blockage of N-glycosylation.* HEK293 cells (0.8 x 10^6) were infected with 4 pfu/cell of recombinant virus vTF7-3 and transfected with NR1 and NR2A genes (4 μg of each plasmid) or NR1 alone as a control (8 μg). After 24 h in the presence of 2 mM DL-AP5, with or without 2 μg/ml tunicamycin (Tn) as indicated, cells were washed and incubated with $^{45}CaCl_2$ together with 2 mM NMDA and 10μM glycine, in the presence or absence of MK-801 (10 μM) and tunicamycin for 5 min. Mean values of the net accumulation of $^{45}Ca^{2+}$ during this time, corresponding to three independent experiments with standard deviations are given. To assess the differences between cells subjected to different treatments, statistic analysis was done with one-way ANOVA plus a Tukey`s post test (***p <0.001).

Figure 2. *Blockade on synthesis of the NR1 but not the NR2A subunits expressed in heterologous systems after inhibition of the N-glycosylation.* **A.** Pulse-labeling of NMDAR subunits. HEK293 cells were infected as before and transfected with NR1 and NR2A genes (4 μg of each plasmid) or control DNA (C) (8 μg). Infection proceeded for 24 h in the presence of 2 mM DL-AP5 and, then, cells were incubated for 3 h in DMEM lacking methionine, with or without 2 μg/ml of tunicamycin (Tn) as indicated. Then, they were pulsed for 30 min with 150 μCi/ml of ^{35}S-methionine in the same medium used for starvation. The effect of N-glycosylation inhibition on the synthesis *de novo* of NMDAR subunits was analyzed by immunoprecipitation (IP) of cell extracts with antibodies specific for NR1 (IPαNR1, upper panel) or conserved regions in the C-termini of NR2A and NR2B subunits (IPαNR2, lower panel), electrophoretic fractionation and autoradiographic detection. The expected mobilities of glycosylated and nonglycosylated (ng) subunits are indicated on the right. Results are representative of four independent experiments. **B.** Effect of short tunicamycin treatment on the steady-state levels of NMDAR subunits. Proteins immunoprecipitated as described were analysed by immunoblotting (IB) using NR1 (IBαNR1) and NR2 (IBαNR2) antibodies as above. **C.** Recognition of in vitro deglycosylated NR1 by specific antibodies.

Protein extracts (30 μg) corresponding to cells infected/transfected as before without tunicamycin were treated with N-glycosidase F (NGlyF) as indicated by the manufacturers or left untreated. Then, immunoprecipitation of NR1 (upper panel) and NR2A (lower panel) was performed as described, and proteins were detected by immunoblotting. The presented results are representative of three independent experiments. D. Effect of the tunicamycin treatment on levels and location of the NMDAR subunits. HEK293 cells were transfected with YFP-NR1 and pME18S-HA-NR2A (0.5 μg of each plasmid) as indicated. After 5 h of transfection, incubation proceeded for additional 24 h with 2 mM DL-AP5, with or without 2 μg/ml of tunicamycin as specified. Double immunofluorescence of permeabilized cells was performed to study levels of the NR1 subunit (green) and NR2A (red). Confocal microscopy images correspond to a single section. Arrows denote regions of subunit co-expression in the plasma membrane of untreated cells. Arrowheads point to aggregates of NR2A found in tunicamycin-treated cells. Results are representative of three independent experiments. The scale bars represent 5 μm.

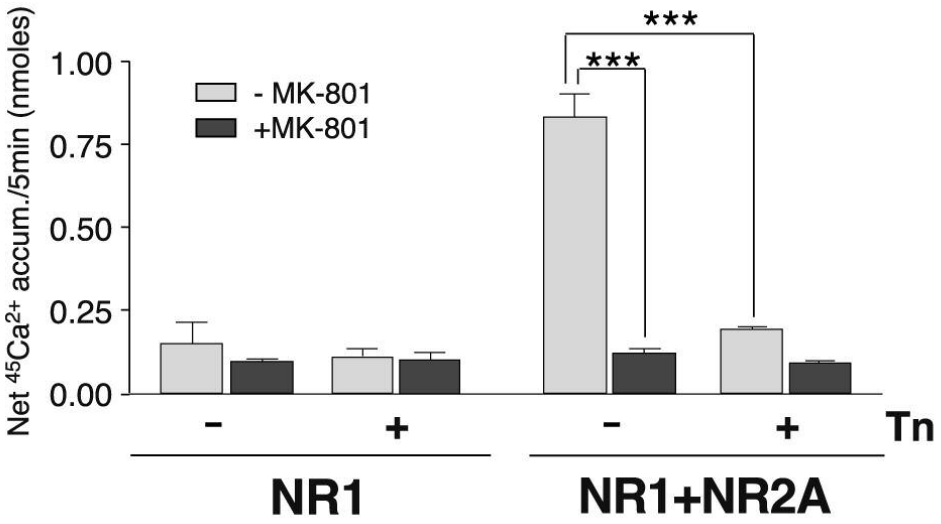
Figure 3. *Blockade on de novo synthesis of NR1 but not NR2 subunits induced by the inhibition of the N-glycosylation in primary cultures of cortical neurons.* **A.** Primary cultures of rat cortical neurons (DIVs 14) were labeled for 4 h with ^{35}S-methionine (150 μCi/ml) in the presence or absence of tunicamycin (2 μg/ml) as indicated, also present 3 h before labeling during methionine starvation. Cell extracts prepared using RIPA buffer containing 0.5% SDS were immunoprecipitated with antibodies specific for the NR1 subunit or NR2, and proteins detected by autoradiography. **B.** Quantitation of newly synthesized NR1 and NR2. Levels of ^{35}S-labeled subunits were established by densitometric analysis of autoradiographies using NIH Image software. Values obtained in cells treated with tunicamycin were referred to those found in untreated cells, arbitrarily given a 100 percent value. Average of three independent experiments with standard deviations is given. To assess the differences, statistic analysis was done with one-way ANOVA plus a Tukey`s post test using Prism v4 software (***p <0.001). Immunoprecipitated proteins as before were analyzed by immunoblot with antibodies specific for the NR2 **(C)** or NR1 **(D)** subunits.

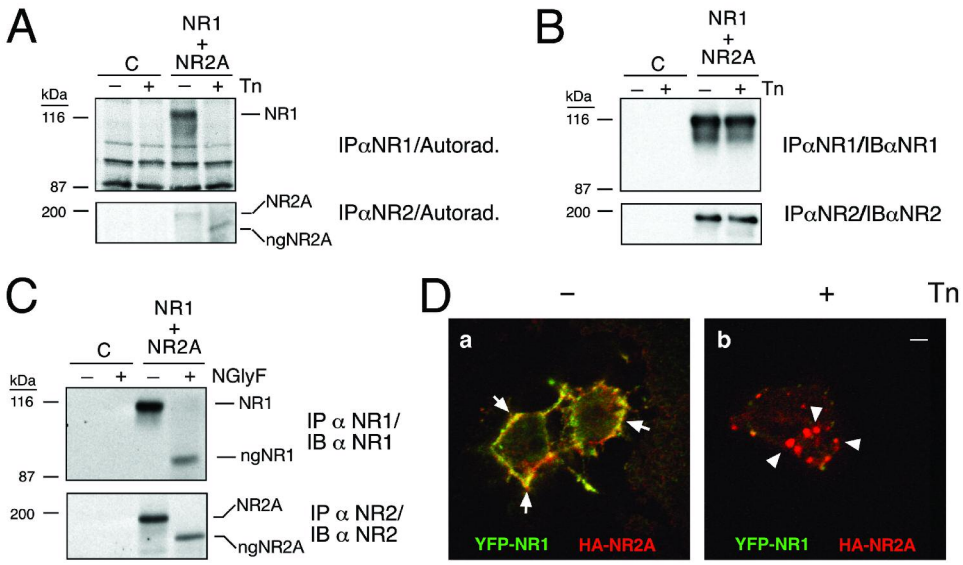
Figure 4. *Disruption of NMDAR assembly induced by inhibition of the N-glycosylation in cortical neurons.* Cultures of cortical neurons were labeled with ^{35}S-methionine as before in the presence of tunicamycin (2 μg/ml) and/or brefeldin A (Bf) (5 μg/ml) as indicated. Cell extracts prepared using standard RIPA buffer (0.1% SDS) were immunoprecipitated using antibodies against the NR1 **(A)** or NR2 subunits **(B)**. Directly immunoprecipitated and interacting proteins were detected by autoradiography. Results are representative of three independent experiments.

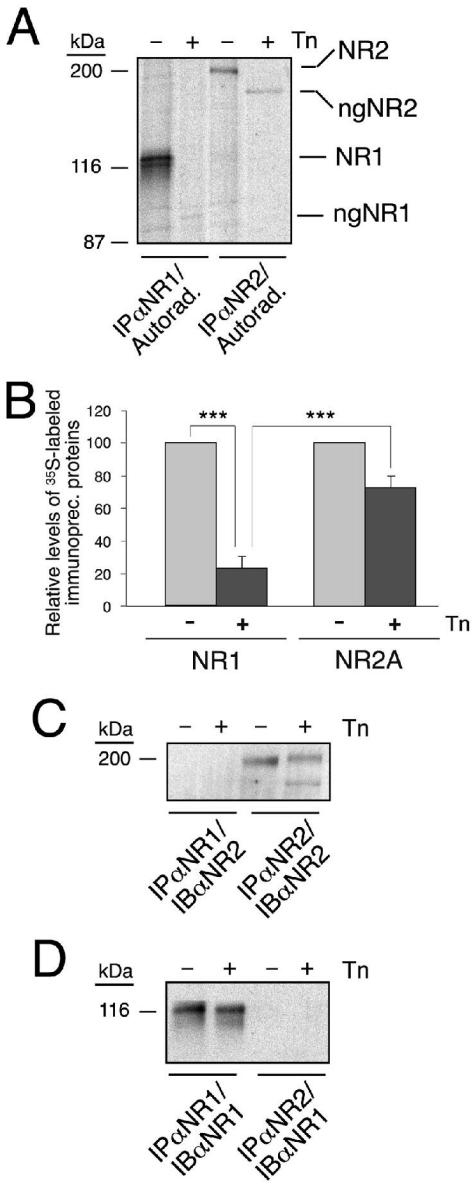
Figure 5. *Induction of ER stress responses by inhibition of N-glycosylation in cultured cortical neurons.* **A.** Primary cultures of cortical neurons (DIVs 14) were incubated with tunicamycin (2 μg/ml) as before for 7 or 24 h or, alternatively, left untreated. Total RNA obtained from these cells was analyzed by Northern blot using ^{32}P-labeled DNA probes specific for NR1, calnexin, BiP/grp78 or CHOP/GADD153 mRNAs. Ethidium bromide staining of total RNA is also shown. **B.** Total protein lysates were prepared from cortical neurons treated with tunicamycin as before and analyzed by immunoblot with antibodies specific for CHOP/GADD153, caspase-12 or calnexin. The presented results are representative of three independent experiments.

Figure 6. *Blockade of the synthesis of NR1 induced by inhibition of the N-glycosylation of cortical neurons is partially due to proteasomal degradation.* **A.** Cultures of rat cortical neurons (DIVs 14) were labeled as before in the presence of tunicamycin (2 μg/ml) and/or the proteasome inhibitor MG132 (50 μM). Cell extracts were immunoprecipitated using antibodies for NR1 (upper panel) or calnexin (lower panel). Immunoprecipitated proteins were detected by autoradiography. **B.** Quantitation of newly synthesized NR1 in cells treated with tunicamycin and MG132. Levels of ^{35}S-labeled subunits were established by densitometric analysis of autoradiographies using NIH Image software. Values obtained were referred to those found in the untreated cells, arbitrarily given a 100 percent value. Average of three independent experiments with standard deviations is given. To assess the differences between cells subjected to different treatments, statistic analysis was done with one-way ANOVA plus a Tukey`s post test using Prism v4 software (***p <0.001). **C.** Proteins immunoprecipitated with the NR1 antibody as above were analyzed by

immunoblot with ubiquitin specific sera. These results are representative of three independent experiments. **D.** Cortical neurons were labeled as described in the presence of tunicamycin (2 μg/ml) and/or the proteasome inhibitors ALLN (50 μM) and lactacystin (Lact; 50 μM), as well as control peptide ALLM (50 μM) as indicated. Cell extracts were prepared and analyzed as before.



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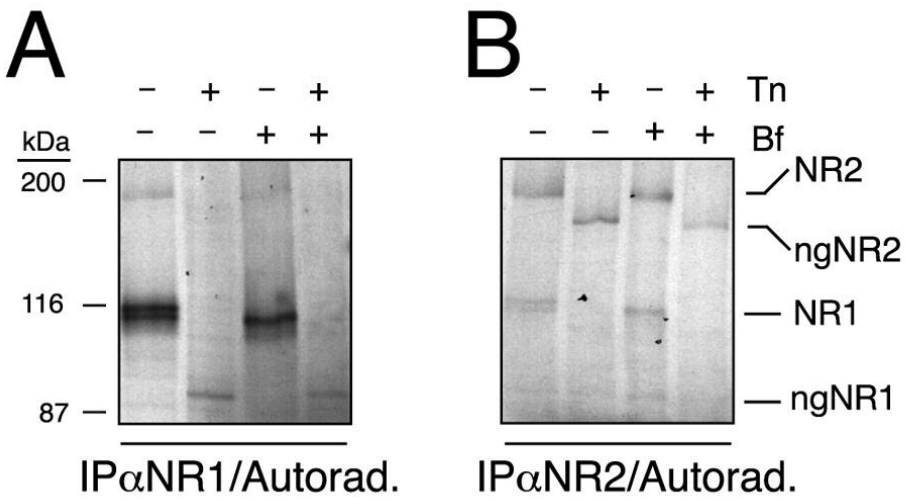

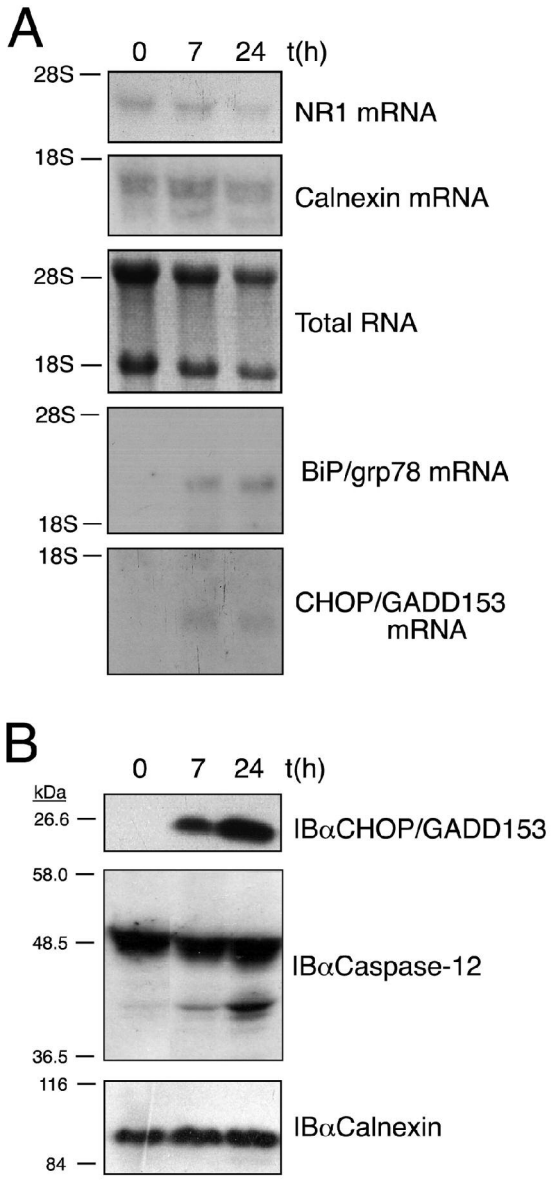
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